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(54) Haptens, immunogens, antibodies and conjugates for 2-oxo-3-hydroxy LSD

(57) The present invention provides a hapten derivatized with a monofunctional or a bifunctional crosslinker, either at the nitrogen of the 8β-carboxamide or at N-6, of 2-oxo-3-hydroxy-LSD.

The present invention also provides an immunogen comprising the aforementioned hapten, coupled to an antigenicity-conferring carrier material, as well as, conjugates comprising the aforementioned hapten covalently bonded to a detectable labelling agent. In addition, the present invention concerns antibodies raised against the aforementioned immunogens.

Finally, the present invention relates to methods and kits for detecting or determining LSD metabolites [2-oxo-3-hydroxy-LSD and N-desmethyl-LSD (nor-LSD)] in biological fluids.

The antibodies of the present invention have broad specificity towards each of 2-oxo-3-hydroxy-LSD and nor-LSD.

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Description

Background

[0001] The present invention relates to haptens that are used for the preparation of immunogens, antibodies and conjugates for use in competitive immunoassays for the detection of the LSD metabolites, 2-oxo-3-hydroxy-LSD and N-desmethyl-LSD (nor-LSD).

[0002] As used hereinafter, the term "LSD metabolite" is intended to embrace 2-oxo-3-hydroxy-LSD and nor-LSD.

[0003] The present invention also relates to a method and kit for detecting or determining LSD metabolites.

[0004] By "detecting" is meant qualitatively analysing for the presence or absence of a substance.

[0005] By "determining" is meant quantitatively analysing for the amount of a substance.

[0006] Lysergic acid diethylamide (LSD) (Figure 1, **1**) is a powerful, psychoactive compound. It is classified as a Schedule I drug. The compound is available in pills, solutions and impregnated sugar cubes, blotting paper or vitamin tablets.

[0007] LSD is a very potent hallucinogen, 10-150 times as potent as psilocybin and 4500-9275 times as potent as mescaline. The isomeric compound d-iso-LSD (Figure 1, **2**) is inactive.

[0008] With increasing restrictions on administration of LSD to human subjects, current knowledge on the distribution, metabolic profile and extraction of LSD in man is limited. A considerable number of reports are available on the distribution and metabolic profile of LSD in animals where the most common metabolites found are nor-LSD (Figure 1, **3**), 2-oxo-3-hydroxy-LSD (Figure 1, **4**), 2-oxo-LSD, 13-hydroxy-LSD, 14-hydroxy-LSD, N-desethyl-LSD, N-ethyl-N-(2-hydroxyethyl)-LSD (amide N), N-ethyl-N-vinyl-LSD (amide N) and lysergic acid. The major metabolites are nor-LSD **3** and 2-oxo-3-hydroxy LSD **4**, the latter of which was only recently detected in human urines submitted for drug testing, its identity having been confirmed by comparing LC-MS characteristics with a reference compound. The average concentration of 2-oxo-3-hydroxy-LSD **4** was 20 times more than that of LSD. In routine analysis of human urines, d-iso-LSD **2** was also detected. This compound is believed to be a by-product from the illicit preparation of LSD.

[0009] Due to the very low dose consumed (usually 40 to 120μg) and due to rapid metabolism with less than 1% excreted unchanged in urine, identification of LSD in biological samples is a major challenge to forensic scientists. Furthermore, the instability of LSD in acid, heat and light has made its identification even more challenging. Because LSD is metabolized to a number of compounds, most known methods are aimed at identifying unchanged LSD in biological samples.

[0010] Although LSD is most commonly detected in urine by GC-MS, immunoassays, particularly competitive binding immunoassays, would be the simplest and most timesaving screening methods available. Competitive binding immunoassays, as their name implies, measure competition in binding to antibody between a fixed amount of labeled antigen, the 'detection reagent' (or conjugate), and an unknown quantity of unlabelled antigen, the 'sample'.

[0011] Commercial immunoassay methods for LSD include radioimmunoassay procedures which are very sensitive, but do require radionuclide tracers, for example ^{125}I and ^{3}H, and, in some cases, a preliminary extraction step. For urine drug testing by radioimmunoassay, samples are identified as positive or negative by comparing the counts with that of a cut-off standard containing 500pg/ml of LSD.

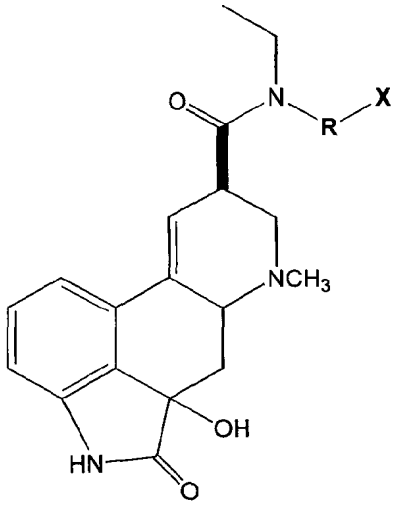
[0012] Nonisotopic homogeneous immunoassays for LSD are also commercially available. The Cloned Enzyme Donor Immunoassay (CEDIA, Boehringer Mannheim) and Enzyme Multiplied Immunoassay (EMIT, Behring Diagnostics) are based on the principle of enzyme activation. The Online Immunoassay (Roche Diagnostic Systems) is based on kinetic interaction of microparticles in solution. These three assays are specially designed for largescale analysis or automated analyzers. Microplate Immunoassay (STC Diagnostics) is available for smallscale testing. These nonisotopic LSD immunoassays correlate well with the original LSD radioimmunoassays.

[0013] All currently commercially available LSD immunoassay methods are specific for the parent drug, LSD, and generally exhibit low cross-reactivity with LSD metabolites. However, Ghoshal *et al* (EP 1 148 339 A2) discloses immunoassay reagents with substantially equal specificity for both LSD and 2-oxo-3-hydroxy-LSD but with low cross-reactivity for nor-LSD (i.e., less than 5%, based on 100% specificity to 2-oxo-3-hydroxy-LSD). The present inventors are unaware of antibodies specific for both 2-oxo-3-hydroxy-LSD and nor-LSD having been reported prior to the present invention, as described herein.

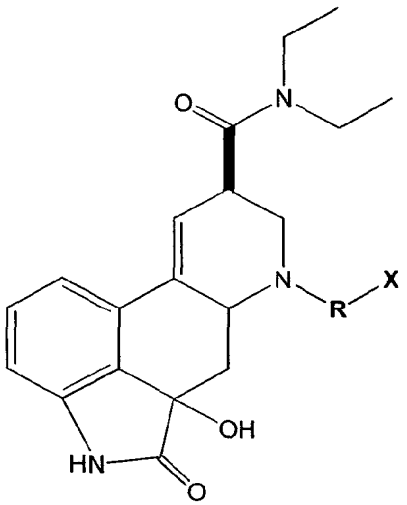
Detailed Description of Invention

[0014] In a first aspect, the present invention provides a hapten derivatised with a monofunctional or a bifunctional crosslinker, either at the nitrogen of the 8β-carboxamide or at N-6, of 2-oxo-3-hydroxy-LSD (Figure 1, **4**).

[0015] Preferably, the hapten has one of the following structural formulae:-



or



in which R is a bivalent link and X is a terminal group, R comprising a substituted or unsubstituted, straight or branched chain, saturated or unsaturated, alkylene moiety, a substituted or unsubstituted, saturated or unsaturated, cycloalkylene moiety or a substituted or unsubstituted arylene moiety; and X comprising a carboxylic acid or an ester thereof, an aldehyde, a thiocarboxylic acid or an ester thereof, preferably thioacetyl, or a halocarboxylic acid or an ester thereof, preferably haloacetyl.

[0016] Thus, in both formulae of the preferred embodiment, the crosslinker comprises -R-X.

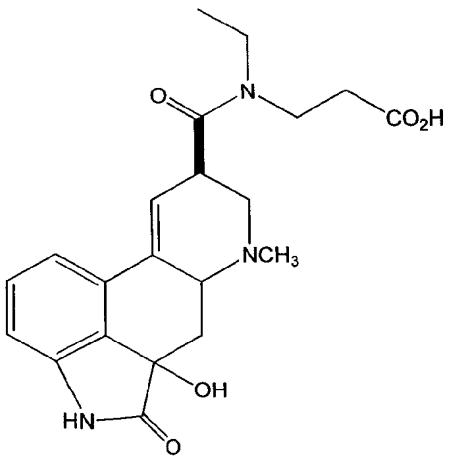
[0017] More preferably, R is a C_{1-6}, most preferably a C_{2-3}, substituted or unsubstituted, straight chain, saturated alkylene moiety.

[0018] Suitable cycloalkylene moieties include cyclohexane.

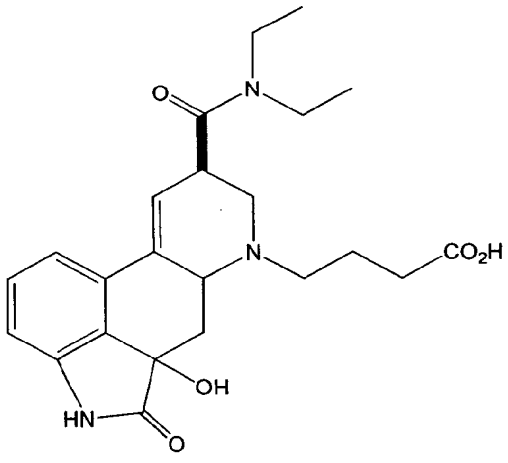
[0019] Suitable arylene moieties include benzene and xylene.

[0020] Advantageously, X is carboxylic acid.

[0021] Most preferably, the hapten is selected from the following hapten derivatives of 2-oxo-3-hydroxy-LSD:



Hapten A



Hapten B

[0022] In Hapten A, R is a saturated, unsubstituted straight chain alkylene group having 2 carbon atoms. In Hapten B, R is a saturated, unsubstituted, straight chain alkylene group having 3 carbon atoms.

[0023] The terminal groups X are used for coupling the haptens of the present invention to carrier materials for the preparation of the corresponding immunogens. The resulting immunogens can be administered to hosts to elicit pro-

duction of avid specific antisera, preferably polyclonal antisera, which are then used to develop sensitive immunoassays for the detection of LSD metabolites.

[0024] The invention, therefore, also provides an immunogen comprising a hapten according to the present invention, coupled to an antigenicity-conferring carrier material. Preferably, the carrier material is a protein, a protein fragment, a synthetic polypeptide or a semisynthetic polypeptide.

[0025] In a further aspect, the present invention concerns antibodies raised against the immunogen of the present invention, the antibodies being capable of binding with at least one structural epitope of 2-oxo-3-hydroxy-LSD. Preferably, the antibodies are fixed on a backing substrate.

[0026] In a still further aspect, the present invention comprises a conjugate comprising the hapten of the present invention covalently bonded to a detectable labelling agent. Preferably, the labelling agent is selected from an enzyme, a luminescent substance, a radioactive substance, or a mixture thereof. More preferably, the labelling agent is an enzyme, preferably a peroxidase, most preferably horseradish peroxidase (HRP). Alternatively, or additionally, the luminescent substance may be a bioluminescent, chemiluminescent or fluorescent material.

[0027] The invention further provides a process of preparing the antibodies, the process comprising the steps of immunising an animal, preferably a vertebrate animal, most preferably a mammalian animal, by repeated administration of an immunogen according to the present invention, and collecting the resulting serum from the immunised animal. Preferably, the process further comprises fixing said serum antibodies to a backing substrate, preferably a solid support, most preferably a polystyrene solid support. Preferably, the antibodies are polyclonal. Alternatively, the antibodies are monoclonal.

[0028] In a further aspect, the present invention comprises a method for detecting or determining LSD metabolites in a sample, the method comprising contacting the sample with the conjugate of the present invention, or a mixture thereof, and with antibodies of the present invention, or a mixture thereof; detecting or determining bound conjugate; and deducing from a calibration curve the presence of, or the amount of, LSD metabolites in the sample.

[0029] In a still further aspect, the invention includes a kit for detecting or determining LSD metabolites, the kit including the conjugate of the present invention, or a mixture thereof; and the antibodies of the present invention, or a mixture thereof. The kit may optionally include instructions for the use of said conjugates and said antibodies for detecting or determining LSD metabolites in a sample.

[0030] Preferably, the sample is a solution, such as a biological fluid. More preferably, the sample is serum or urine.

[0031] In the method and kit of the present invention, it is preferred that the respective crosslinkers (of the immunogen and the conjugate) are different.

[0032] In a further aspect, the present invention involves use of the conjugates according to the present invention, or a mixture thereof, with the antibodies according to the present invention, or a mixture thereof, to detect or determine LSD metabolites in samples such as biological fluids.

[0033] The focus of the present invention is the preparation of antibodies specific to both of the LSD metabolites, 2-oxo-3-hydroxy-LSD (Figure 1, **4**) and nor-LSD (Figure 1, **3**). By this is meant, the antibodies should have a cross-reactivity for nor-LSD of more than 50%, preferably more than 75%, when compared to 100% for 2-oxo-3-hydroxy-LSD, and *vice versa.* In order to achieve such broad specificity, haptens A and B are generated by derivatization of 2-oxo-3-hydroxy-LSD at the N-carboxamide and nor positions (N-6).

Preparation of Haptens

[0034] Hapten A was prepared in three steps from lysergic acid (see Figure 2 of the accompanying drawings). The carboxylic group of lysergic acid **5** was activated with 1,1'-carbonyldiimidazole (CDI) in dimethylformamide (DMF). The activated ester intermediate obtained was reacted with N-ethyl N-(2-carbethoxy)ethyl amine **10** (prepared by reaction of ethyl amine with ethyl acrylate) to yield ester **6**. Oxidation of the double bond of the indole ring of the tartrate salt of ester **6** with calcium hypochlorite, generated *in situ* by the action of chlorine gas on calcium hydroxide, produced the ester 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carbethoxy)ethyl amide **7.** Hapten A was obtained after saponification of ester **7** by aqueous potassium hydroxide in tetrahydrofuran.

[0035] Hapten B was prepared in three steps from nor-LSD **3** (see Figure 3 of the accompanying drawings). N-alkylation of nor-LSD **3** with ethyl 4-bromobutyrate in the presence of sodium hydride in DMF produced ester **8**. Oxidation of the double bond of the indole ring of ester **8** was performed using the same conditions used for oxidation of ester **6,** to produce the ester 2-oxo-3-hydroxy-6-(3-carboethoxy)propyl-nor-LSD **9.** Hapten B was obtained after saponification of **9** using aqueous potassium hydroxide in tetrahydrofuran.

Preparation of Immunogens and Conjugates

[0036] Although the haptens of the present invention provide defined structural epitopes, they are not in themselves immunogenic and therefore need to be conjugated to a carrier material which will elicit an immunogenic response when

administered to a host animal. Suitable carrier materials commonly contain poly(amino acid) segments and include polypeptides, proteins such as albumins, serum proteins e.g. globulins and ocular lens proteins and lipoproteins. Illustrative protein carrier materials include bovine serum albumin (BSA), egg ovalbumin, bovine gamma globulin, thyroxine binding globulin, keyhole limpet haemocyanin (KLH) etc. Alternatively, synthetic poly(amino acids) having a sufficient number of available amine groups such as lysine may be employed, as may other synthetic or natural polymeric materials bearing reactive functional groups. In particular, carbohydrates, yeasts or polysaccharides may be conjugated to the haptens of the present invention to produce immunogens of the present invention.

[0037] Each hapten of the present invention can also be coupled to a labelling agent such as an enzyme (for example, horse radish peroxidase), a substance having fluorescent properties or a radioactive label for the preparation of conjugates (or detection reagents) for use in the immunoassays. The fluorescent substance may be, for example, a monovalent residue of fluorescein or a derivative thereof.

[0038] In preparing immunogens or conjugates with haptens of the present invention where a thiol group is present, i.e., where X is a thiocarboxylic acid or an ester thereof, maleimide, halo or vinylsulphone groups must first be introduced to the carrier material or labelling agent (enzyme or label) using heterobifunctional linkers such as: N-(γ-maleimidobutyryloxy)succinimide ester (GMBS); succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC); (m-maleimidobenzoyl)-N-hydroxysuccinimide (MBS); succinimidyl 4-(*p*maleimidophenyl)butyrate (SMPB); N-succinimidyl (4-iodoacetyl)aminobenzoate (SIAB); bromoacetylglycine N-hydroxysuccinimide; N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP); or vinylsulphone (Pierce Chemical Company, USA). The thus-modified carrier material or labelling agent can then be conjugated via the thiol groups on the hapten. For haptens without a thiol group present, such as haptens **A** and **B** of the present invention, conjugation is performed without prior-modification of the carrier material or labelling agent using standard methods of conjugation such as mixed anhydride, EDC or succinimidyl activation of the hapten.

[0039] In order to confirm that adequate conjugation of hapten to carrier material has been achieved, prior to immunization, each immunogen is evaluated using matrixassisted UV laser desorption /ionization time-of-flight mass spectroscopy (MALDI-TOF MS). In the case of the preferred carrier material, bovine serum albumin, a minimum of 6 molecules of hapten per carrier molecule is preferred. Each of the immunogens of the present invention can be used for immunization, in order to produce antibodies of the present invention.

General Procedure for MALDI-TOF Analysis of Immunogens.

[0040] MALDI-TOF mass spectrometry was performed using a Voyager STR Biospectrometry Research Station laserdesorption mass spectrometer coupled with delayed extraction. An aliquot of each sample to be analysed was diluted in 0.1% aqueous trifluoroacetic acid (TFA) to create 1mg/ml sample solutions. Aliquots (1μl) were analysed using a matrix of Sinapinic acid and bovine serum albumin (Fluka) was used as an external calibrant. Figure 5 of the accompanying drawings shows the analysis for BSA carrier material. As will be seen, a major signal was present which indicates an average protonated mass for this sample of m/z 66,400.

[0041] The signal at m/z 33,202 is consistent with the major component in a doubly-charged form.

Preparation of Antisera

[0042] In order to generate polyclonal antisera, each immunogen of the present invention is mixed with Freund's Adjuvant and the mixture is injected into a host animal, such as a rabbit, sheep, mouse, guinea pig or horse. Further injections (boosts) are made and serum is sampled for evaluation of antibody titer. When the optimal titer has been reached, the host animal is then bled to yield a suitable volume of specific antiserum. The degree of antibody purification required depends on the intended application. For many purposes, there is no requirement at all for purification, however, in other cases, such as where the antibody is to be immobilized on a solid support, purification steps can be taken to remove undesired material and eliminate non-specific binding.

[0043] The specific antibodies of the present invention are useful as reagents in biochemical assays for the detection or determination of LSD metabolites in biological fluids.

[0044] In the following Examples, percentages are to be taken as percentages (volume/volume), unless otherwise specified.

Example 1: Synthesis of N-ethyl N-(2-carbethoxy)ethyl amine linker 10

[0045] To 110ml of a 2M solution of ethyl amine in tetrahydrofuran (THF) at 0°C was added dropwise ethyl acrylate (8.0ml, 73.92mmol) in 40ml of tetrahydrofuran (THF). The reaction mixture was stirred overnight at room temperature. The reaction solution was filtered through a cotton wool plug and concentrated under reduced pressure to yield the title compound **10** (9.01g, 84%) as a clear liquid.

[0046] υ_{max}/cm^{-1} 3319.68, 2969.10, 1732.07, 1191.87 (FT-IR : Figure 8)

Example 2: Synthesis of lysergic acid N-ethyl N-(2-carbethoxy)ethyl amide 6

[0047] A mixture of lysergic acid **5** (934mg, 3.27mmol) in 40ml of dry dimethylformamide (DMF) was treated with 1,1'-carbonyldiimidazole (CDI, 795mg, 4.91mmol) and stirred under nitrogen at room temperature for 1 hour. The N-ethyl N-(2-carbethoxy)ethyl amine linker **10** (1.90g, 13.08mmol) in 10ml of dry dimethylformamide (DMF) was added dropwise and the reaction solution was stirred overnight at room temperature. The reaction solution was concentrated under reduced pressure and the resulting residue was dissolved in 200ml of chloroform. The chloroform solution was washed twice with 100ml of water, dried over anhydrous sodium sulphate, and concentrated under reduced pressure to yield the crude title compound 6 (2.2g) as an oil. This was used without further purification in the following Example.

[0048] υ_{max}/cm^{-1} 3121.90, 2987.10, 2848.65, 1735.30, 1676.97, 1202.97 (FT-IR : Figure 9)

Example 3: Synthesis of 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carbethoxy)ethyl amide 7

[0049] To a mixture of tartaric acid (2.52g, 16.79mmol) dissolved in 40ml of water and cooled in ice, was added 2.2g of crude lysergic acid N-ethyl N-(2-carbethoxy)ethyl amide **6** (1.29g, 3.27mmol; based on 100% yield from the previous Example). A solution of calcium hypochlorite was prepared by dissolving chlorine gas (1.55g, 21.83mmol) into a solution of calcium hydroxide (0.81g, 10.93mmol) in 240ml of water.

The cloudy solution was passed through a 0.45μM membrane filter, to remove any undissolved material, and cooled in ice. To 170ml of this freshly prepared calcium hypochlorite solution was added the lysergic acid N-ethyl N-(2-carbethoxy)ethyl amide tartrate solution. The reaction was stirred at 0°C-5°C for 30 min. The reaction mixture was diluted with 80ml of saturated sodium bicarbonate solution and extracted with 6x100ml of chloroform. The chloroform extracts were combined, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The resulting residue was chromatographed on silica gel to give 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carbethoxy)ethyl amide **7** (385mg, 28%) as an amorphous dark brown solid (R_f 0.53 on silica using 25% methanol in chloroform as eluent).

[0050] υ_{max}/cm^{-1} 3180.04, 2980.66, 2937.48, 2803.85, 1731.59, 1669.11, 1448.37, 1385.34 (FT-IR : Figure 10)

Example 4: Synthesis of 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carboxy)ethyl amide (Hapten A)

[0051] To a mixture of 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carbethoxy)ethyl amide **7** (63.6mg, 0.15mmol) in 5ml of tetrahydrofuran (THF) and 5ml of water was added solid potassium hydroxide (12.5mg, 0.22mmol). The reaction was stirred at room temperature for 3 hours (h) until the reaction was complete by TLC analysis. The reaction solution was neutralised to pH 7 using 1N HCl and concentrated to dryness under reduced pressure. The residue was dissolved in a 10% methanol in chloroform mixture and the inorganic salts were removed by filtration. Evaporation of the solvents yielded 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carboxy)ethyl amide **(Hapten A)** (45mg, 76%) as an amorphous brown solid (R_f 0.52 on silica using 20% methanol in chloroform as eluent) .

[0052] υ_{max}/cm^{-1} 3297, 2963, 1723, 1557, 1099, 1020, 802 (FT-IR)

Example 5: Conjugation of 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carboxy)ethyl amide to BSA (Immunogen A)

[0053] To a solution of 2-oxo-3-hydroxy lysergic acid N-ethyl N-(2-carboxy)ethyl amide **(Hapten A),** (45mg, 0.11mmol) in 1ml of dimethylforamide (DMF) was added dicyclohexylcarbodiimide (DCC) (27.9mg, 0.14mmol) and N-hydroxysuccinimide (NHS) (15.6mg, 0.14mmol) and the mixture was stirred at room temperature overnight. The dicyclohexylurea formed was filtered off and the solution was added dropwise to a solution of BSA (150mg) in 6ml of 0.05M sodium bicarbonate solution (pH 8.5). The mixture was stirred overnight at 4°C, protected from light. The solution was then dialysed overnight against 5L of PBS (pH 7.2) at 4°C and freeze-dried.

[0054] By MALDI-TOF (see Figure 6 of the accompanying drawings), a major signal was present which indicates an average protonated mass for this sample of m/z 70,351. The signal at m/z 35,248 is consistent with the major component in a doubly-charged form. These data suggest that an average of 9.8 molecules of Hapten A have been conjugated per molecule of BSA.

Example 6: Synthesis of 6-cyano-nor-LSD

[0055] To a refluxing solution of cyanogen bromide (2.96g, 27.95mmol) in 150ml of dry chloroform, under nitrogen, was added dropwise a solution of lysergic acid diethylamide **1** (2.00g, 6.19mmol) in 100ml of dry chloroform. The reaction was heated under reflux for 4 hours and cooled to room temperature. The organic phase was extracted twice

with 150ml of a 1% (w/v) tartaric acid solution. The combined aqueous washes were extracted with 100ml of chloroform and the combined organic phases were dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The black residue was chromatographed on silica gel using ethyl acetate to yield 6-cyano-nor-LSD (879mg, 42%) as a pale yellow solid after evaporation of the solvent.

[0056] υ_{max}/cm^{-1} 3192, 2933, 2212, 1636, 1449, 986

Example 7: Synthesis of nor-LSD 3

[0057] A mixture of 6-cyano-nor-LSD (879mg, 2.63mmol) in 8ml of acetic acid and 2ml of water, under nitrogen, was treated with zinc dust (1.53g, 23.39mmol) and heated under reflux for 6h. The solution was allowed to cool and decanted off from the excess zinc with a water wash. The reaction solution was concentrated to a small volume under reduced pressure and the concentrate was diluted with 10ml of water. The pH of the solution was taken to pH 9 using concentrated ammonia solution at 0°C. The resulting gummy precipitate was extracted four times with 50ml of chloroform. The combined chloroform extracts were dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield nor-LSD **3** (625mg, 77%) as a light brown amorphous solid (R_f 0.47 on silica using 20% methanol in chloroform as eluent).

[0058] υ_{max}/cm^{-1} 3260, 2974, 2934, 1620, 1447

Example 8: Synthesis of 6-(3-carboethoxy)propyl-nor-LSD 8

[0059] A mixture of nor-LSD **3** (625mg, 2.02mmol) in 5ml of dry dimethyl formamide (DMF), was treated with ethyl bromobutyrate (290µL, 2.02mmol), potassium carbonate (839mg, 6.06mmol) and a catalytic amount of potassium iodide, and stirred under nitrogen at 40°C overnight. The reaction solution was concentrated to a residue under reduced pressure and chromatographed on silica gel using 5% methanol in chloroform to yield 6-(3-carboethoxy)propyl-nor-LSD **8** (368mg, 43%) after evaporation of the solvents (R_f 0.77 on silica using 20% methanol in chloroform as eluent).

[0060] υ_{max}/cm^{-1} 3268.56, 2975.19, 2934.49, 1731.00, 1623.51 (FT-IR : Figure 11)

Example 9: Synthesis of 2-oxo-3-hydroxy-6-(3-carboethoxy)propyl-nor-LSD 9

[0061] To a mixture of tartaric acid (196mg, 1.31mmol) dissolved in 20ml of water, cooled in ice, was added 6-(3-carboethoxy)propyl-nor-LSD **8** (368mg, 0.87mmol). A calcium hypochlorite solution was prepared by dissolving chlorine gas (1.55g, 21.83mmol) into a solution of calcium hydroxide (0.81g, 10.93mmol) in 240ml of water. The cloudy solution was passed through a 0.45µM membrane filter, to remove any undissolved material, and cooled in ice. To 45ml of the freshly prepared calcium hypochlorite solution was added the 6-carboethoxypropyl-lysergic acid diethylamide tartrate solution and the reaction was stirred at 0°C-5°C for 30 min. The reaction was diluted with 80ml of saturated sodium bicarbonate solution and extracted with 6x100ml of chloroform. The chloroform extracts were combined, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. The resulting residue was chromatographed on silica gel to give 2-oxo-3-hydroxy-6-(3-carboethoxy)propyl-nor-LSD **9** (102mg, 26%) as a brown solid (R_f 0.44 on silica using 10% methanol in chloroform as eluent).

[0062] υ_{max}/cm^{-1} 3242.29, 2976.74, 1727.98, 1615.25, 1447.09, 1214.64 (FT-IR : Figure 12)

Example 10: Synthesis of 2-oxo-3-hydroxy-6-(3 carboxy)propyl-nor-LSD (Hapten B)

[0063] To a mixture of 2-oxo-3-hydroxy-6-(3-carboethoxy)propyl-nor-LSD **9** (90mg, 0.20mmol) in 3 ml of tetrahydrofuran (THF) and 3 ml of water was added solid potassium hydroxide (22mg, 0.40mmol). The reaction was stirred at room temperature for 3h until the reaction was complete by TLC analysis. The reaction solution was neutralised to pH 7 using 1N HCl and concentrated to dryness under reduced pressure. The residue was dissolved in a 10% methanol in chloroform mixture and the inorganic salts were removed by filtration. Evaporation of the solvents yielded 2-oxo-3-hydroxy-6-(3-carboxy)propyl-nor-LSD (**Hapten B**), (86mg, 102%) as a foamy brown solid.

[0064] υ_{max}/cm^{-1} 3242.47, 2979.73, 1720.79, 1605.03 (FT-IR : Figure 13)

Example 11: Conjugation of 2-oxo-3-hydroxy-6-(3-carboxy)propyl-nor-LSD to BSA (Immunogen B)

[0065] To a solution of 2-oxo-3-hydroxy-6-(3-carboxy)propylnor-LSD **(Hapten B)** (32mg, 0.08mmol) in 0.5ml of dimethylforamide (DMF) was added dicyclohexylcarbodiimide (DCC) (18.5mg, 0.09mmol) and N-hydroxysuccinimide (NHS) (10.4mg, 0.09mmol) and the mixture was stirred at room temperature overnight. The dicyclohexylurea formed was removed by filtration. The solution obtained was added dropwise to a solution of BSA (100mg) in 6ml of 0.05M sodium bicarbonate solution (pH 8.5). The mixture was then stirred overnight at 4°C protected from light. The solution

was dialysed against 5L of phosphate buffered saline (PBS) (pH 7.2) at 4°C for 24 hours (2 changes) and then freeze-dried.

[0066] By MALDI-TOF (see Figure 7 of the accompanying drawings), a major signal was present which indicates an average protonated mass for this sample of m/z 69,699. The signal at m/z 34,862 is consistent with the major component in a doubly-charged form. These data suggest that an average of 8.1 molecules of Hapten B have been conjugated per molecule of BSA.

Example 12: Preparation of antibodies to Immunogens A and B, prepared in Examples 5 and 11.

[0067] An aqueous solution of each of the immunogens prepared in Examples **5** and **11** was formulated with Freund's Complete Adjuvant (FCA) to form an emulsion consisting of 2mg/ml immunogen in 50% (v/v) FCA. Three sheep were immunized with this emulsion, 0.25ml being subcutaneously injected at each of four sites in the flank of each animal. Subsequent immunizations (boosts) contained 1mg/ml immunogen emulsified in 50% (v/v) Freund's Incomplete Adjuvant (FIA) and were administered in the same manner at monthly intervals for 1 year. Blood sampling took place 7 to 14 days after each boost. Each sample was processed to produce antiserum which was further purified by caprylic acid and ammonium sulfate precipitation to yield an immunoglobulin G (IgG) fraction. The IgG fraction was evaluated by competitive ELISA microtitre plate assay, as described below.

Example 13 : Development of competitive ELISAs for 2-oxo-3-hydroxy-LSD

[0068] The wells of an enhanced binding 96 well polystyrene microtiter plate were coated with the IgG fraction of the antiserum raised to an immunogen of the present invention, diluted in 10mM Tris, pH8.5 (125µl/well). The appropriate antibody coating dilution was determined using standard ELISA chequerboard techniques. The plate was incubated for 2 hours at 37°C, washed 4 times with Tris buffered saline containing Tween 20 (TBST) and tapped dry. Standard solutions of 2-oxo-3-hydroxy-LSD were prepared in TBST at 0, 10, 50, 100, 500, 1000, 2500 and 5000 pg/ml and 25µl of each was added to the appropriate wells (Figure 4). Conjugates (detection reagents) of the present invention were diluted in Tris buffer (pH7.2) containing EDTA, D-mannitol, sucrose, thimerosal and BSA, appropriate dilutions being determined by standard ELISA chequerboard techniques, and 100µl of each was added to the appropriate wells (Figure 4). The plate was incubated at 37°C for 2 hours. Excess unbound conjugate was removed by washing 6 times over a 10 minute period with TBST. 125µl of tetramethylbenzidine (TMB) substrate solution was added to each well of the plate which was then incubated for 15 to 20 minutes in the dark at room temperature. The reaction was terminated by addition of 125µl 0.2M H_2SO_4 to each well. The absorbance was then measured at 450nm using a microtiter plate reader.

Example 14 : Cross reactivity of the 2-oxo-3-hydroxy-LSD competitive ELISA with LSD and its metabolites

[0069] Standard solutions of LSD and its metabolites were prepared in TBST at 0, 10.0, 50.0, 100.0, 500.0, 1000, 2500 and 5000 pg/ml. Employing each series of standards in the present competitive ELISAs, calibration curves were generated and these were used to determine the cross-reactivity of the immunoassays with LSD and its metabolites, cross-reactivity being calculated according to the following formula:

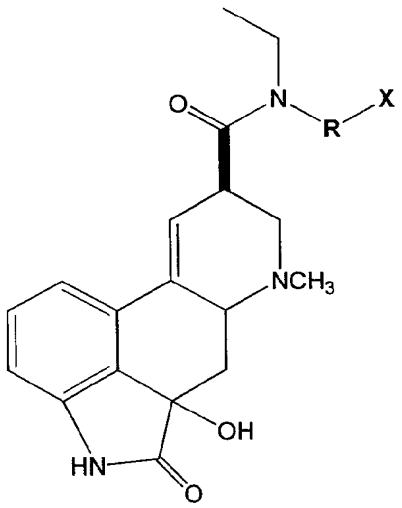
$$\%CR = IC50_{2\text{-}oxo}/IC50_{LSD} \times 100$$

where %CR is the percentage cross-reactivity, $IC50_{2\text{-}oxo}$ is the concentration of 2-oxo-3-hydroxy-LSD that causes 50% displacement of signal and $IC50_{LSD}$ is the concentration of LSD or LSD metabolite that causes 50% displacement of signal.

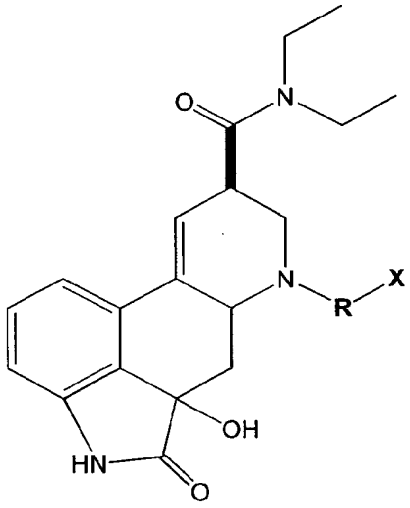
Claims

1. A hapten derivatised with a monofunctional or a bifunctional crosslinker, either at the nitrogen of the 8β-carboxamide or at N-6, of 2-oxo-3-hydroxy-LSD.

2. A hapten according to Claim 1, having one of the following structural formulae:-



or

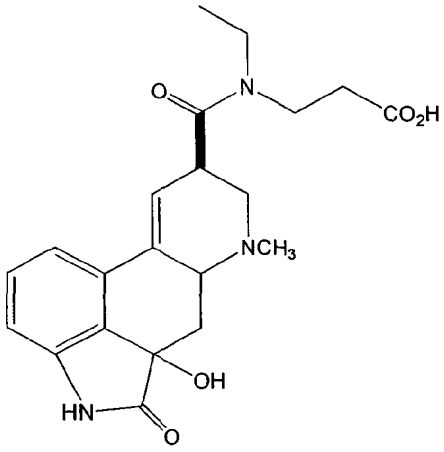


in which R is a bivalent link and X is a terminal group, R comprising a substituted or unsubstituted, straight or branched chain, saturated or unsaturated, alkylene moiety, a substituted or unsubstituted, saturated or unsaturated, cycloalkylene moiety or a substituted or unsubstituted arylene moiety; and X comprising a carboxylic acid or an ester thereof, an aldehyde, a thiocarboxylic acid or an ester thereof, preferably thioacetyl, or a halocarboxylic acid or an ester thereof, preferably haloacetyl.

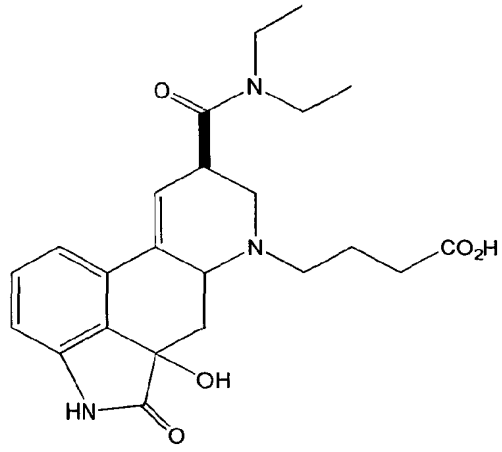
3. A hapten according to Claim 2, in which R is a C_{1-6}, most preferably a C_{2-3}, substituted or unsubstituted, straight chain, saturated alkylene moiety.

4. A hapten according to Claim 2 or 3, in which X is carboxylic acid.

5. A hapten according to Claim 2, the hapten being selected from the following hapten derivatives of 2-oxo-3-hydroxy-LSD:



Hapten A



Hapten B

6. A hapten according to Claim 5, in which, in Hapten A, R is a saturated, unsubstituted straight chain alkylene group having 2 carbon atoms.

7. A hapten according to Claim 5, in which, in Hapten B, R is a saturated, unsubstituted, straight chain alkylene group having 3 carbon atoms.

8. An immunogen comprising a hapten according to any one of Claims 1-7, coupled to an antigenicity-conferring carrier material.

9. Antibodies raised against the immunogen of Claim 8, the antibodies being capable of binding with at least one structural epitope of 2-oxo-3-hydroxy-LSD.

10. A conjugate comprising the hapten of any one of Claims 1-7, covalently bonded to a detectable labelling agent.

11. A conjugate according to Claim 10, in which the labelling agent is selected from an enzyme, a luminescent substance, a radioactive substance, or a mixture thereof.

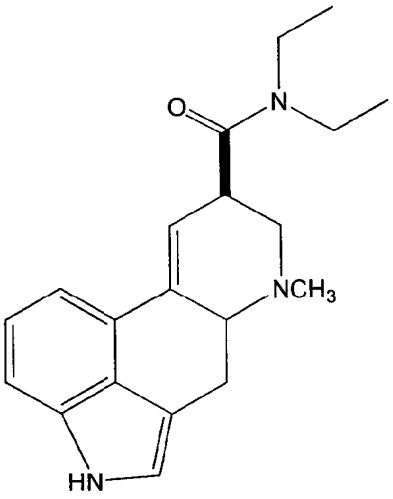
12. A process of preparing the antibodies according to Claim 9, the process comprising the steps of immunising an animal, preferably a vertebrate animal, most preferably a mammalian animal, by repeated administration of an immunogen according to Claim 8, and collecting the resulting serum from the immunised animal.

13. A method for detecting or determining LSD metabolites in a sample, the method comprising contacting the sample with the conjugate of Claim 10 or 11, or a mixture thereof, and with antibodies of Claim 9, or a mixture thereof; detecting or determining bound conjugate; and deducing from a calibration curve the presence of, or the amount of, LSD metabolites in the sample.

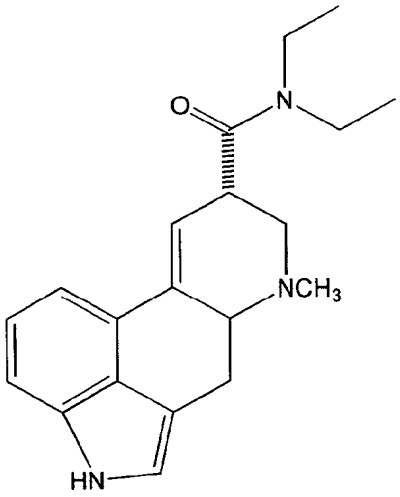
14. A kit for detecting or determining LSD metabolites, the kit including the conjugate of Claim 10 or 11, or a mixture thereof; and the antibodies of Claim 9, or a mixture thereof.

15. Use of the conjugates according to Claim 10 or 11, or a mixture thereof, with the antibodies according to Claim 9, or a mixture thereof, to detect or determine LSD metabolites in samples such as biological fluids.

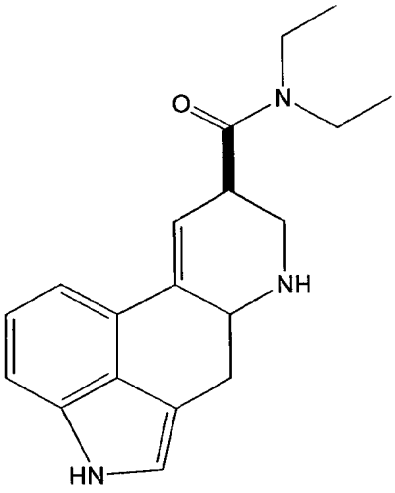
Figure-1. Structure of LSD and related compounds



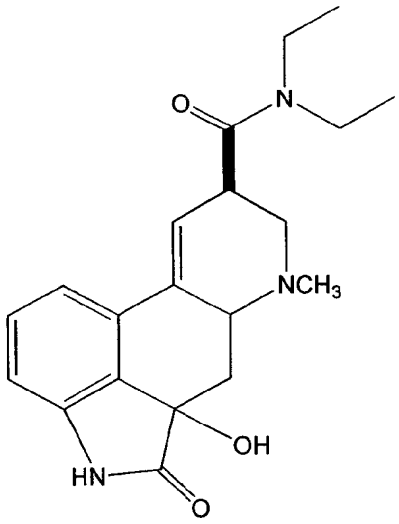
LSD 1



iso-LSD 2

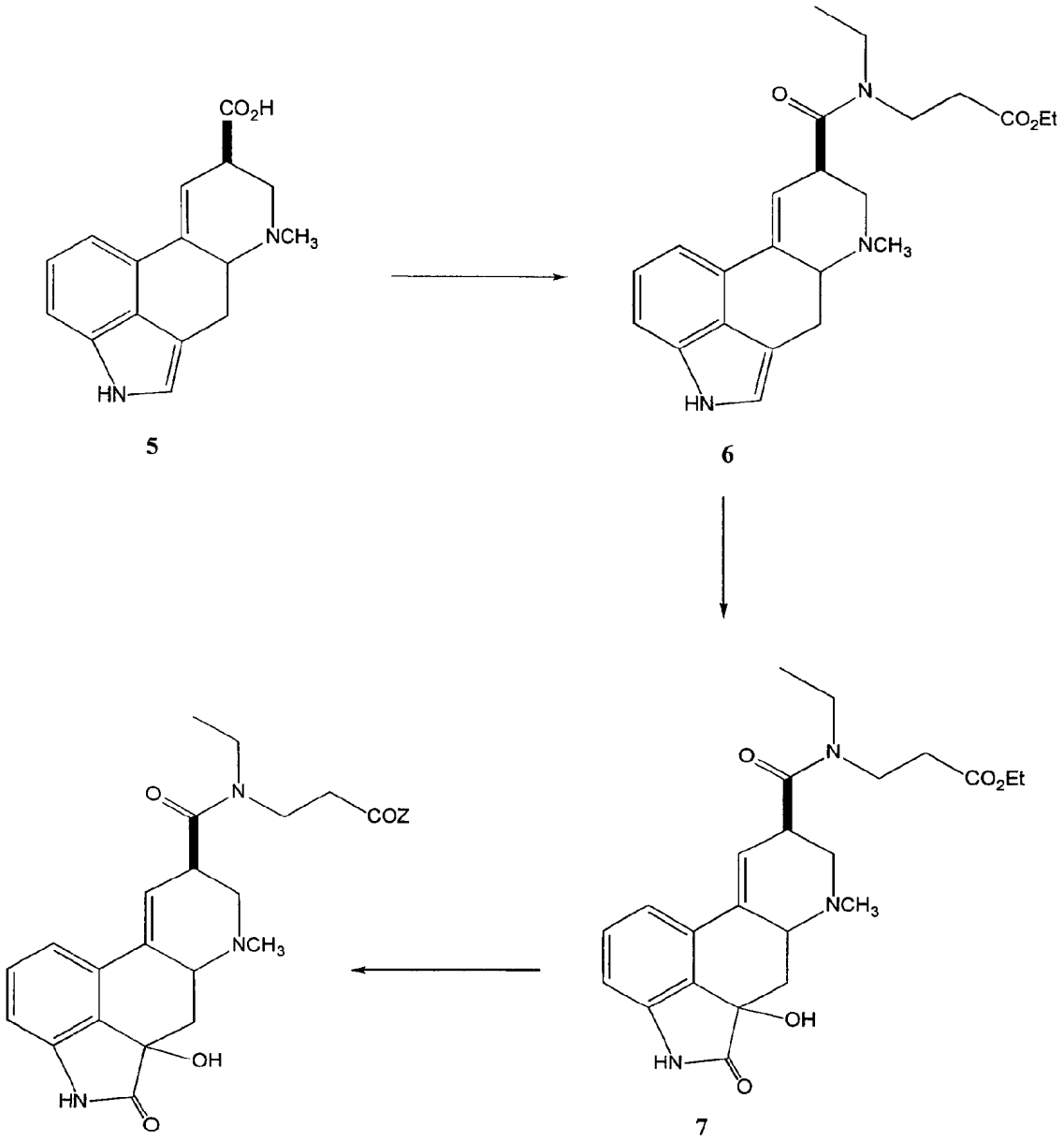


nor-LSD 3



2-oxo-3-hydroxy-LSD 4

Figure-2 Hapten A and immunogen A for 2-oxo-3-hydroxy LSD



Z = OH, Hapten A
Z = NHBSA, Immunogen A

Figure-3 : Hapten-B and Immunogen B for 2 -oxo-3-hydroxy LSD

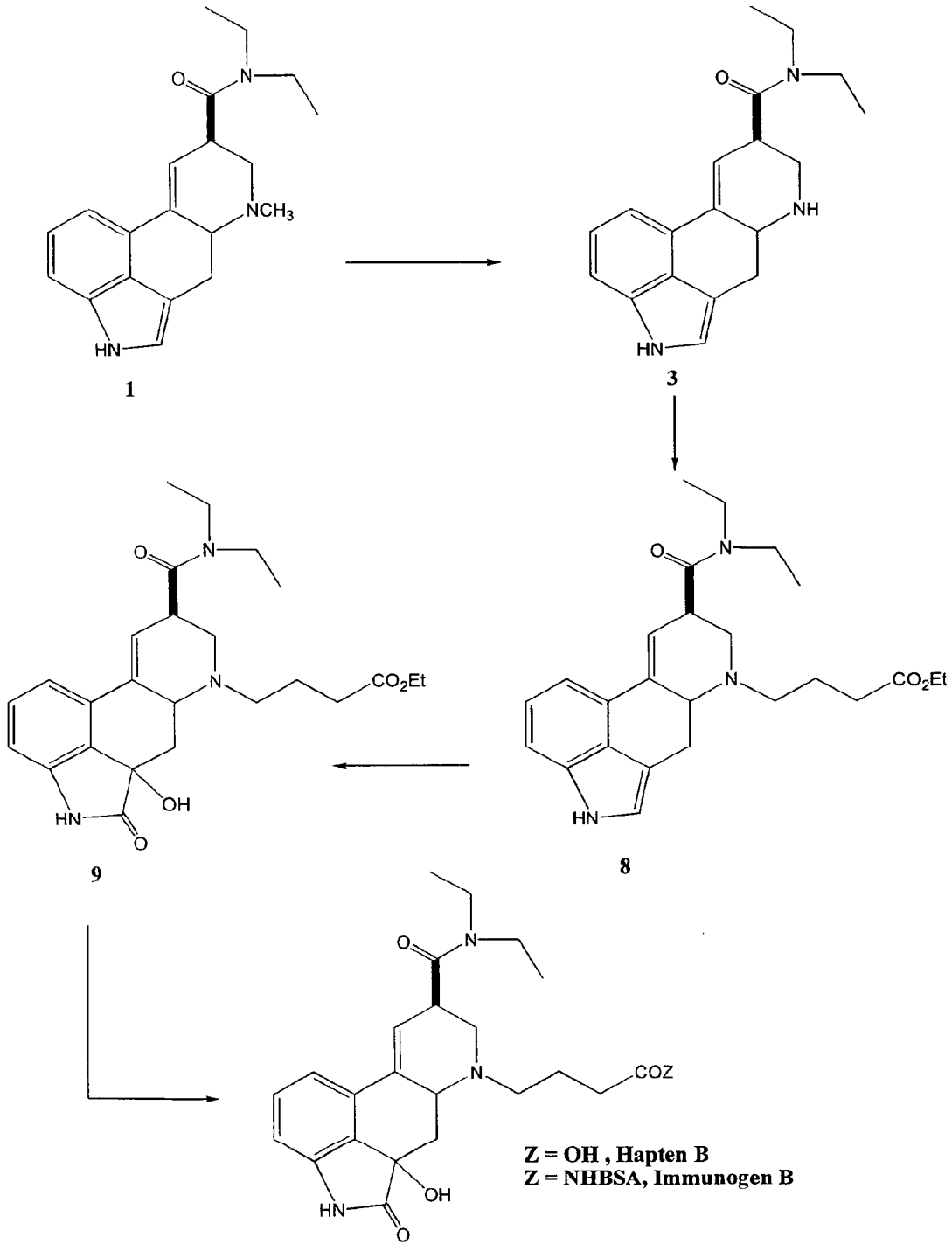
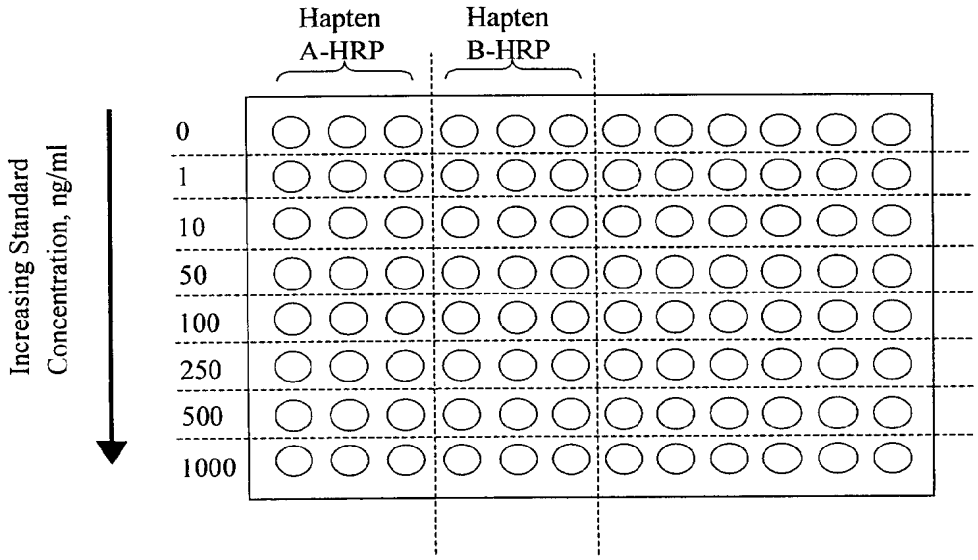


Figure - 4: Competitive ELISA microtiter plate assays for 2-oxo-3-hydroxy-LSD



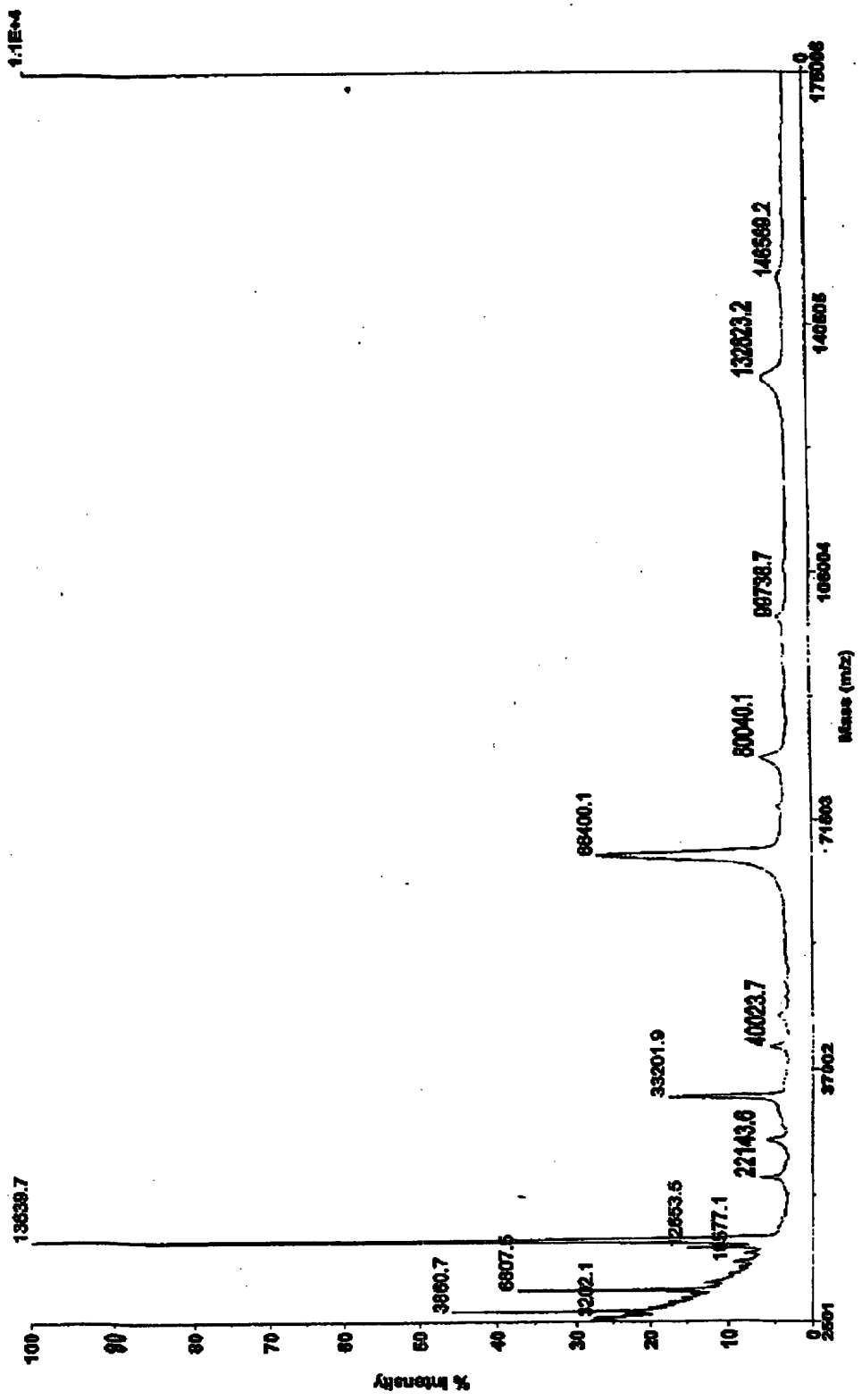


Figure 5: BSA Carrier Material

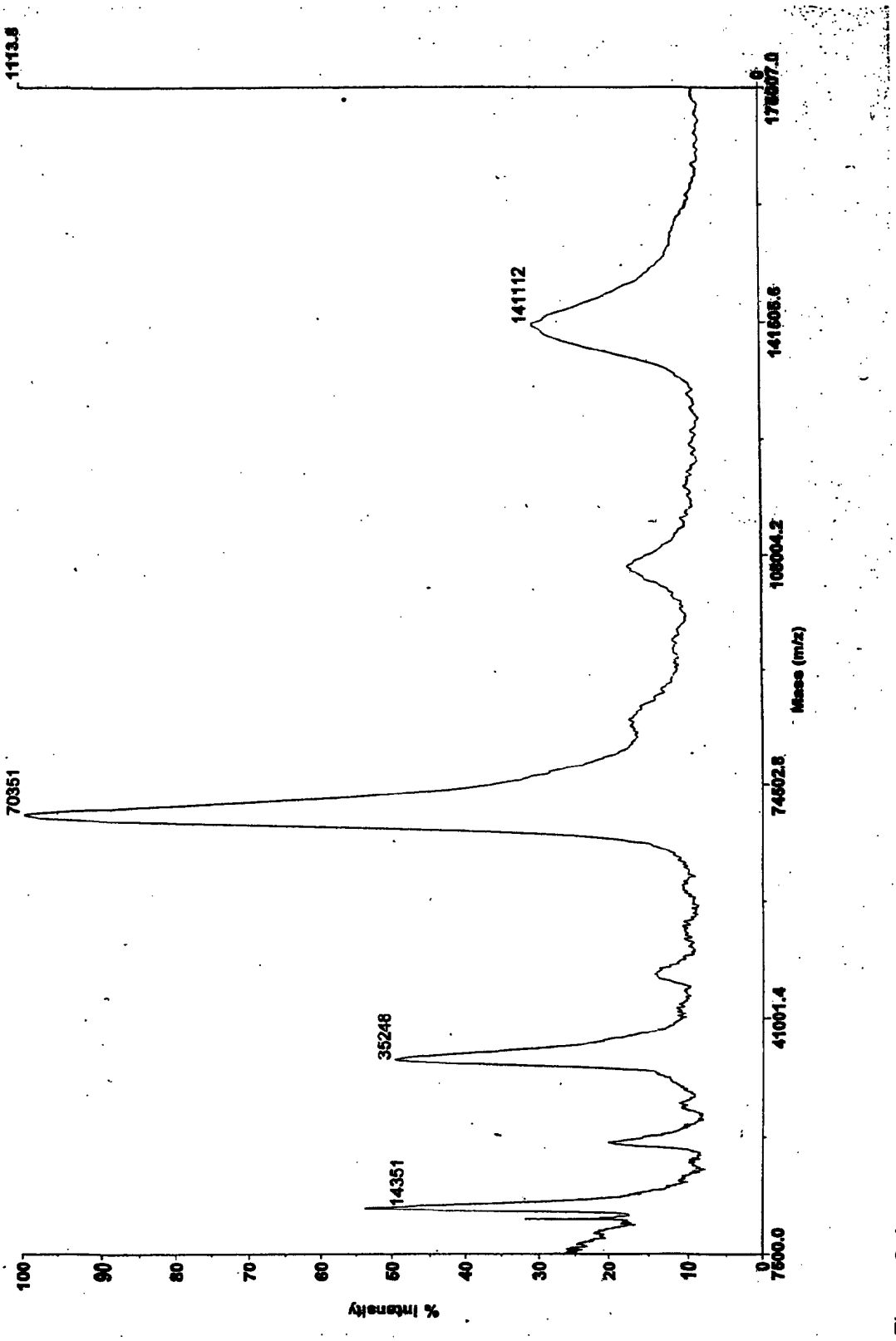


Figure 6: Immunogen A

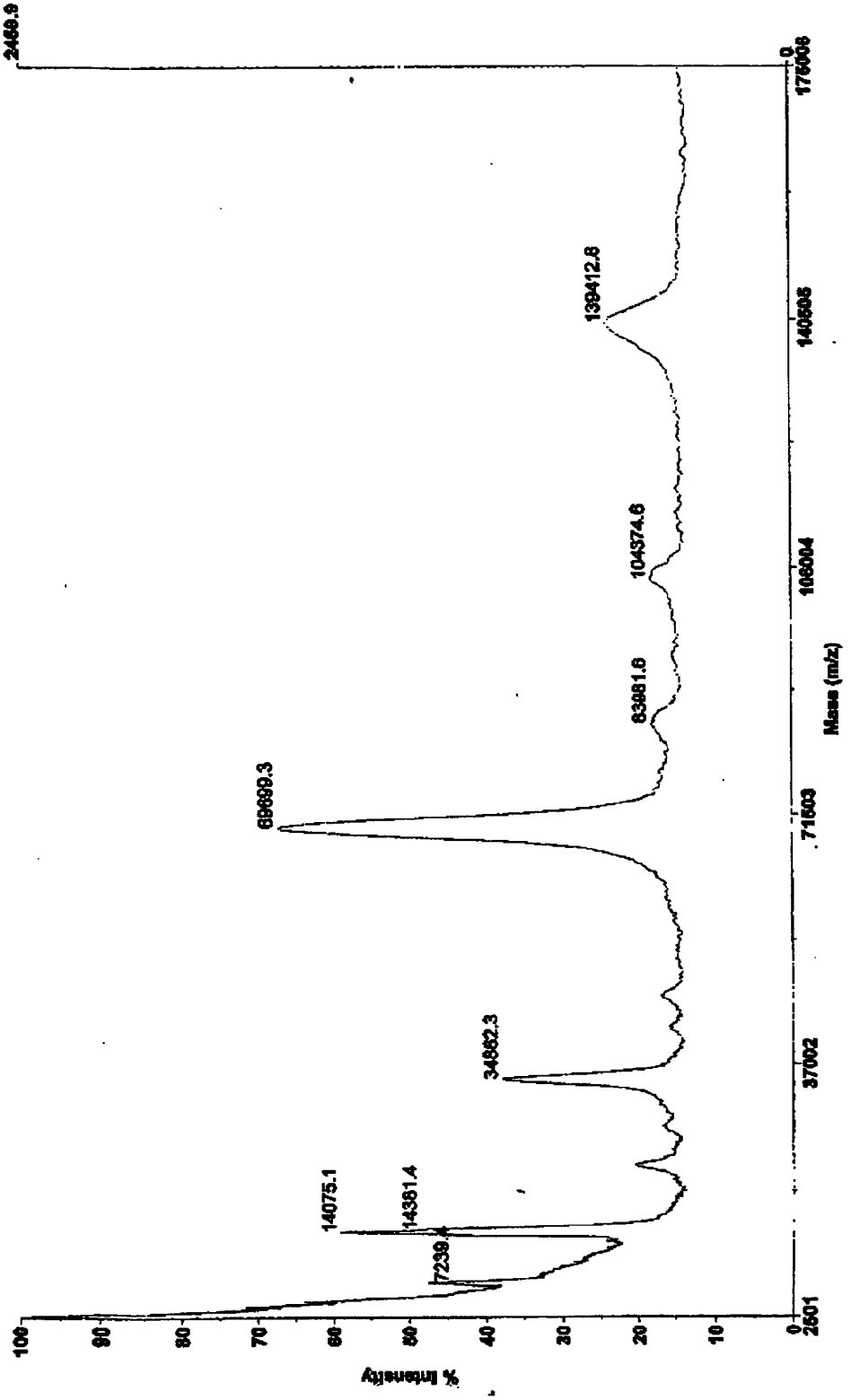
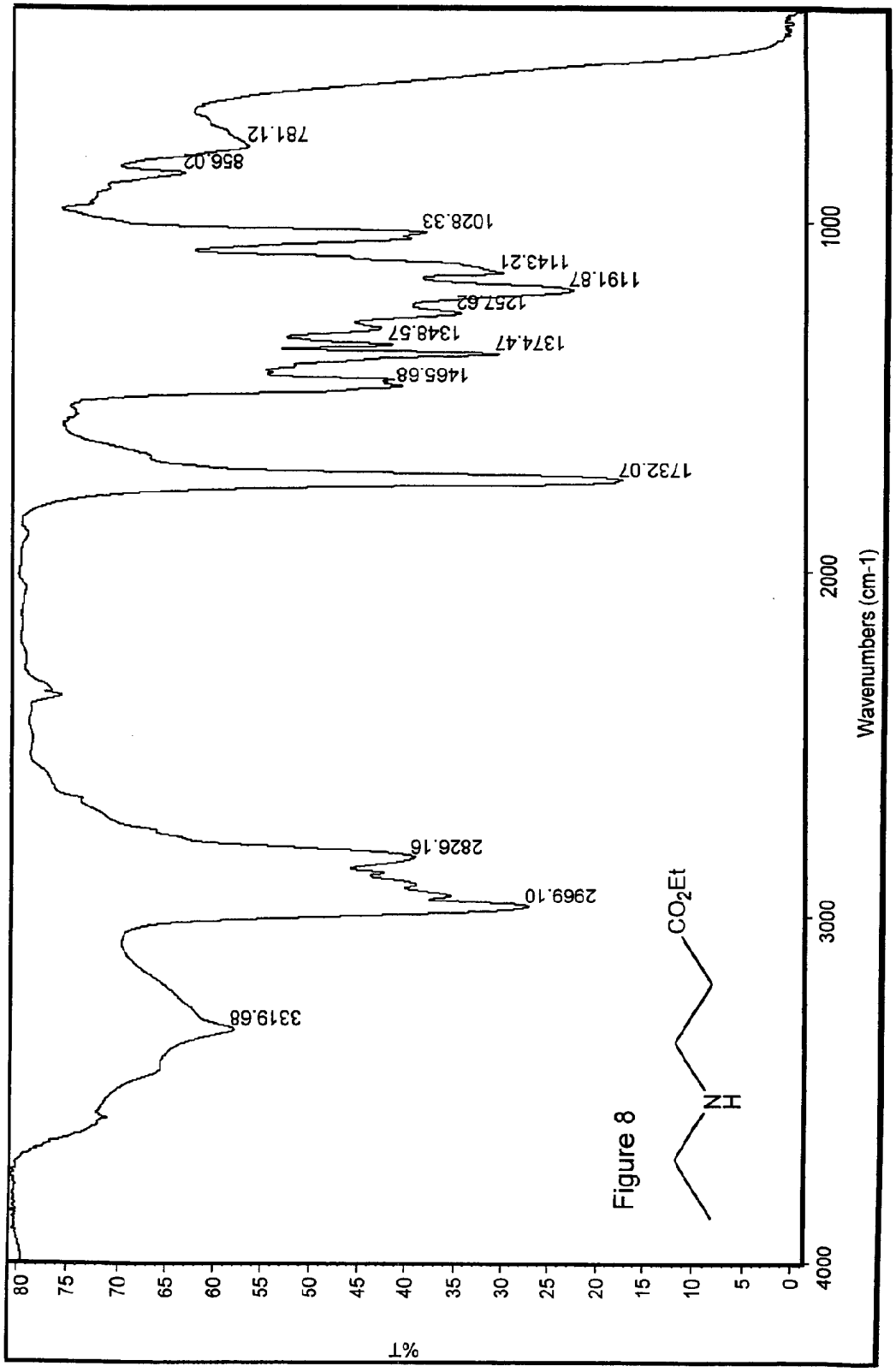


Figure 7: Immunogen B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60
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70
75
80
3319.68
2969.10
2826.16
1732.07
1465.68
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1028.33
856.02
781.12
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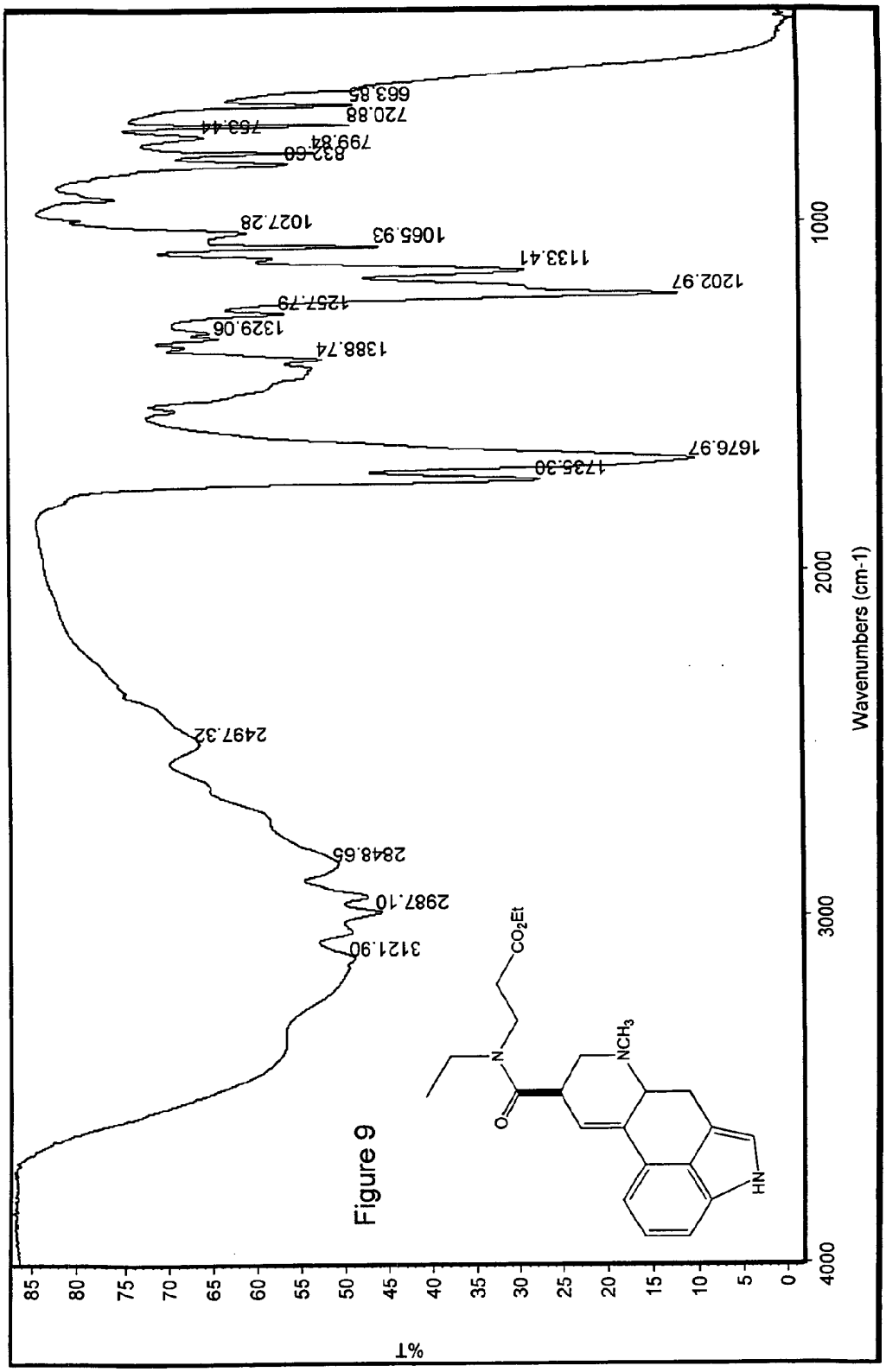
NH

Figure 8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3121.90
2987.10
2848.65
2497.32
1735.30
1676.97
1388.74
1329.06
1257.79
1202.97
1133.41
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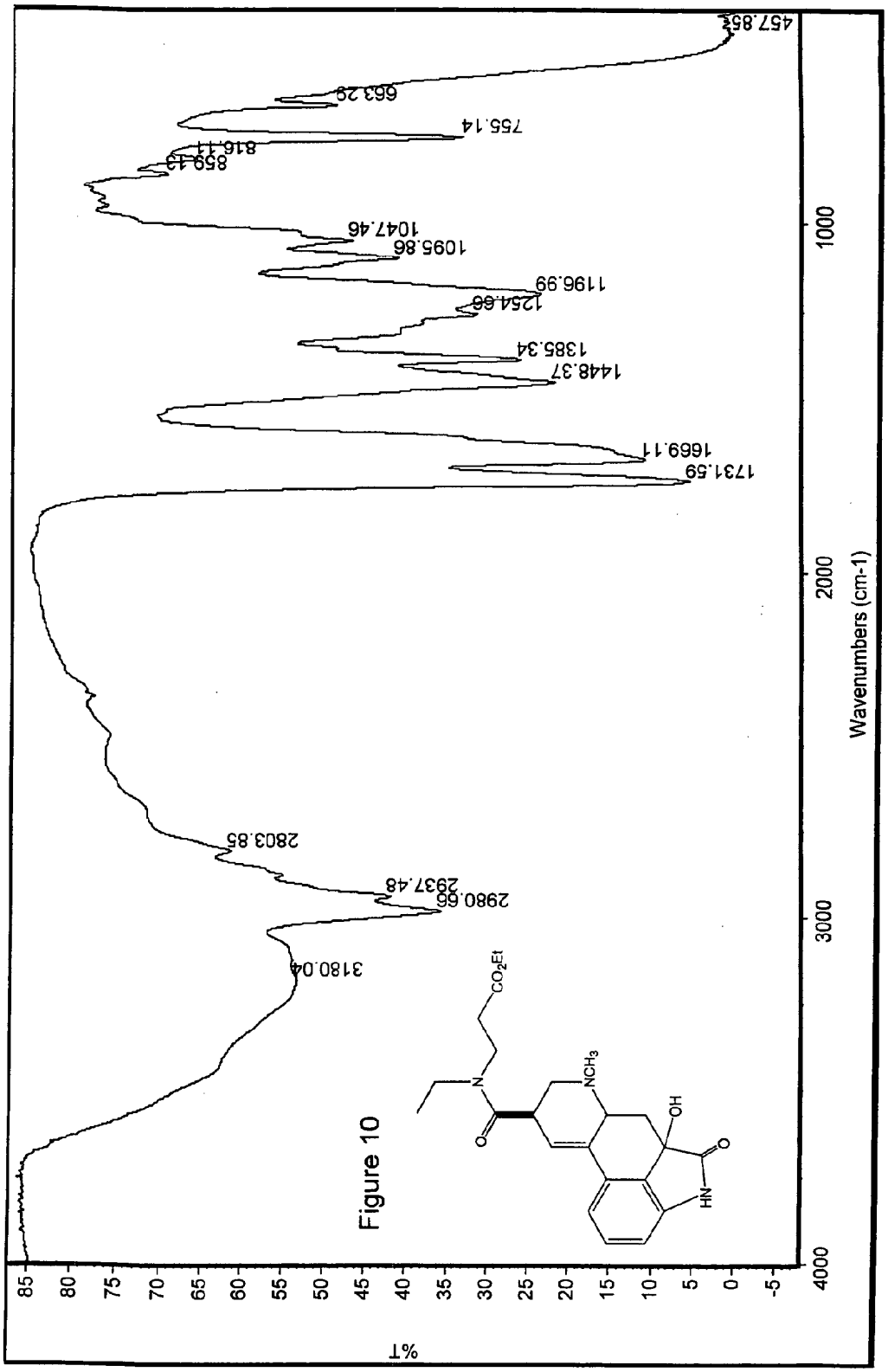
NCH3
N
O
HN

Figure 9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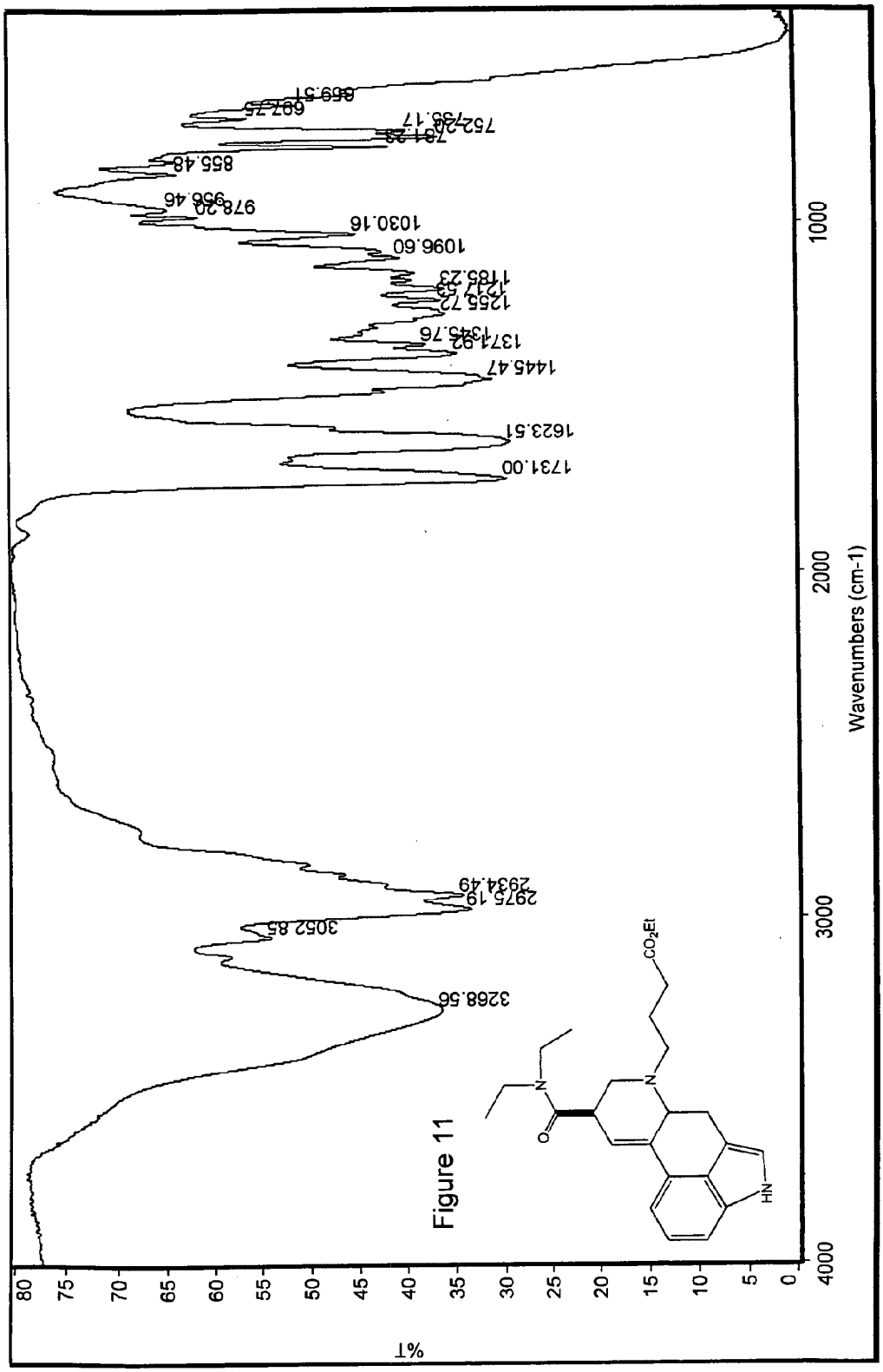
NCH3
OH
O
HN

Figure 10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1731.00
1623.51
1445.47
1374.92
1345.76
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1183.23
1096.60
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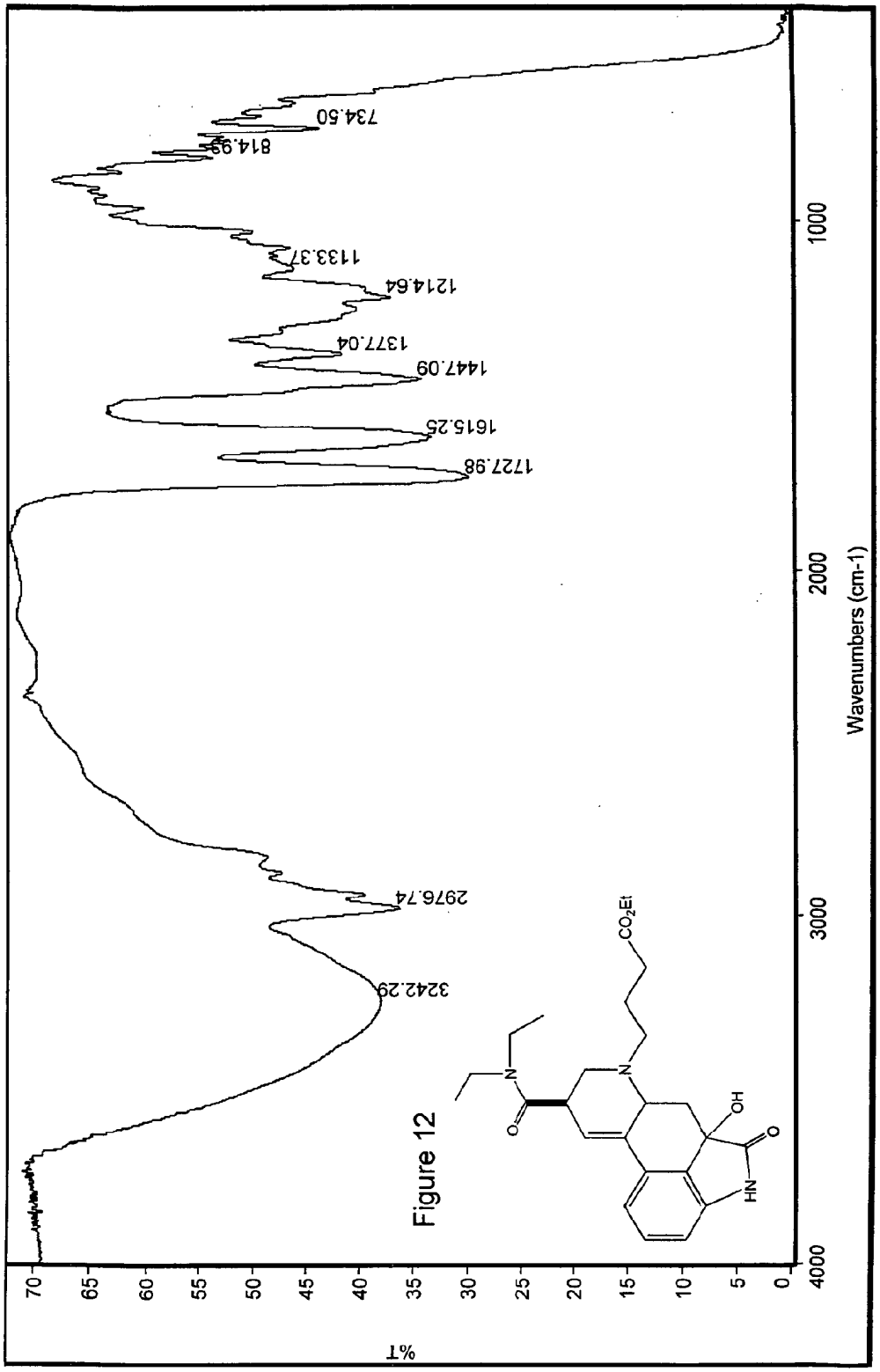
Figure 11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Wavenumbers (cm-1)
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2976.74
1727.98
1615.25
1447.09
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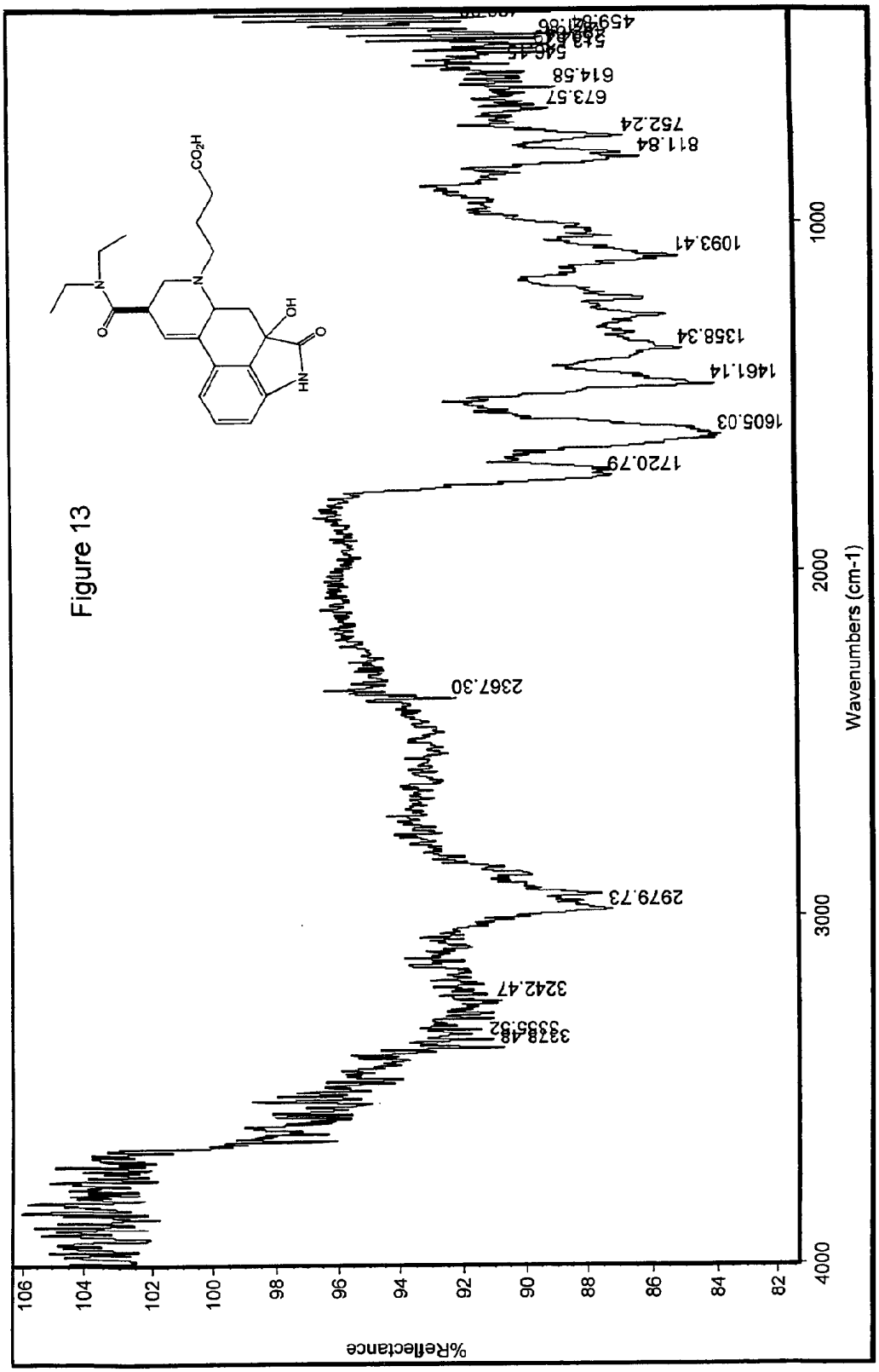
814.99
734.50
CO2Et
OH
O
O
N
N
HN

Figure 12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98
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94
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90
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86
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82
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3000
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1000
Wavenumbers (cm-1)
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3335.92
3242.47
2979.73
2367.30
1720.79
1605.03
1461.14
1358.34
1093.41
811.84
752.24
673.57
614.58
OH
NH
O
N
CO2H

Figure 13

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 02 08 0687

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Y	US 6 207 396 B1 (MORRILL PAUL R ET AL) 27 March 2001 (2001-03-27) * abstract; claims; figures * ---	1-15	A61K31/48 A61K39/385 C07K16/00 C07K16/44
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X	LI ZHUYIN ET AL: "New synthesis and characterization of (+)-lysergic acid diethylamide (LSD) derivatives and the development of a microparticle-based immunoassay for the detection of LSD and its metabolites" BIOCONJUGATE CHEMISTRY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, US, vol. 8, no. 6, November 1997 (1997-11), pages 896-905, XP002175870 ISSN: 1043-1802 * page 897 - page 902; figures * ---	1-15	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C07D G01N A61K C07K
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	28 April 2003	GONCALVES M L F C

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